SUBSTRATE EVALUATION AND OPTIMIZATION FOR SURFACE-ENHANCED RAMAN SPECTROSCOPY OF BACTERIA

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ABSTRACT

The threat of biological agents to soldiers and the civilian community was amply demonstrated in the fall of 2001. The feasibility of using surface-enhanced Raman spectroscopy (SERS) to detect and identify bacteria is being evaluated. In order to use SERS for bacterial detection and identification, it is necessary to determine the most appropriate type of SERS substrate to use. We are examining gold colloids in suspension, immobilized gold colloids, electrochemically roughened gold, periodic particle arrays (PPA), and film over nanosphere substrates (FONS). Briefly, PPA's are prepared by depositing gold or silver in the interstitial spaces in a close-packed array of polystyrene nanospheres, while FONS are prepared by depositing approximately half a nanosphere diameter of gold or silver on top of a close-packed array of polymer nanospheres. We are evaluating each of these substrate types to determine which will have a high affinity for bacteria, whether we need to modify the surface of the substrate to attract bacteria, and the degree to which each type of substrate enhances the Raman scattering from the bacterial targets. At this point, our initial examination of gold colloids immobilized on glass surfaces has yielded mixed results.

INTRODUCTION

Detection and identification of chemical and biological agents at sufficiently low levels to protect troops and the civilian population is a critical challenge to currently fielded systems. The range of hazardous materials also includes toxic industrial chemicals (TICs) and materials (TIMs). These materials must be detected before they can do harm, so the detection methods must be extremely sensitive. The problem is complicated by the need to detect the target materials against large backgrounds of structurally similar, innocuous components of the environment. Spectroscopic methods provide a high probability of accomplishing this task.

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Raman spectroscopy is an excellent method for unique chemical identification. Raman scattering is an inelastic scattering process that yields information about the vibrational energy levels of a molecule or assembly of molecules and is complementary to IR spectroscopy. However, IR spectroscopy is poorly suited to aqueous samples, due to the broad water absorption bands in the IR region while Raman scattering is easily accomplished in water. The peaks in the Raman spectrum of a material also tend to be narrower than those in its IR absorption spectra, which aids in identification due to less spectral overlap between peaks. Finally, Raman spectroscopy can be performed at wavelengths where common telecommunications fiber optics can be implemented, rather than expensive, hygroscopic IR fibers. The applicability of Raman spectroscopy to bacterial identification and analysis has been demonstrated.[1] The major detriment of normal Raman spectroscopy is the inherent weakness of the scattering process, limiting applicability to trace analysis.

Methods to enhance the sensitivity of Raman scattering have been known for over 25 years.[2] Surface-enhanced Raman scattering (SERS) is a well-known method for improving the signal level in Raman scattering. Enhancements of 10 to 16 orders of magnitude have been reported for aggregates of colloidal metal particles. One of the mechanisms that result in SERS is the magnification of the local electric field at nanometer scale features that are illuminated by light of the appropriate wavelength. The field enhancement is a strongly localized effect, extending out from the surface for a small fraction of one wavelength (<100 nm). SERS has been used to detect the presence of chemical agents and their degradation products for water quality monitoring. Sylvia, *et al.* has developed a reproducible method for preparation of electrochemically roughened SERS substrates for their aqueous CW agent monitoring system.[3] Optimization of the SERS substrate and conditions for bacterial identification will be necessary for our particular application. A major part of the optimization process will be developing coatings or surface layers that will ensure that the target materials will move into the enhanced field region for SERS without adding an unacceptable background signature.

The primary competing technologies are DNA and antibody tests for biological materials and ion mobility spectroscopy (IMS) for chemical agents. There are many variations on DNA hybridization assays and antibody tests including flow cytometry, surface plasmon resonance detection, ELISA, and sandwich assays. Each of these biological detection methods requires *a priori* knowledge of the targets, in order to have the sensing substrate prepared with the appropriate antibodies or oligonucleotide hybridization target strands. IMS is the current fielded technique for chemical agent detection. FTIR has been recently demonstrated in combination with neural networks for the identification of genus, species, and in one case, even strain of bacteria.[4] In comparison with these methods, SERS has the advantages of providing more flexibility in the number of agents we can simultaneously identify, the ability to classify unknowns of similar structure to known threats, and the ability to simultaneously detect and identify chemical and biological threats. In addition, SERS spectra can be obtained in aqueous media, unlike FTIR spectra.

There are a number of different methods available to prepare SERS substrates. The first reports of SERS were from electrode surfaces.[5] Further study indicated that roughness features on the order of several to tens of nanometers in size are responsible for the enhanced Raman scattering signal. Other studies have used colloidal metal particles, especially Au and Ag, to obtain SERS spectra. Recently, efforts have focused on providing better control of the substrate that either of these methods provide. One recent report utilizes surface immobilized colloids for SERS.[6] An alternative is provided by the periodic particle arrays developed by Prof. Richard van Duyne from Northwestern University.[7] We are currently investigating each of these types of substrate and our preliminary results are shown here.

EXPERIMENTAL METHODS

The optical train used in these experiments has been described previously.[8] Briefly, it consists of a Kr or Ar-Kr mixed gas laser operating at 647.1 nm, a fiber optic probe for light delivery and collection, and a monochromator with an array detector for spectral collection.

Ten and twenty nm unconjugated colloidal gold solutions [Lot numbers 40K9150 and 49H92651 respectively] were purchased directly from Sigma and 40 and 60 nm unconjugated colloidal gold solutions [Lot numbers 500326 and 508076 respectively] were purchased from Polysciences, Inc. Dynamic light scattering was used to verify the particle size and distribution stated by the manufacturer. The colloids were used directly without any other modification. The colloid solution was mixed with an aqueous suspension of the bacteria by adding 0.500 mL of the concentrated washed bacteria to 2.500 mL of gold colloid.

Immobilized colloid substrates have been prepared following the procedure of Olson, et al.[6] Specifically, quartz microscope slides were soaked in piranha solution (4 parts concentrated H_2SO_4 and 1 part 30% H_2O_2) at 60 °C for 10 minutes to fully hydrolyze the surface. The slides were then rinsed with methanol and oven dried at 110 °C for approximately 30 minutes than allowed to cool in a desiccator. The slides were then individually placed in 50 mL plastic Digitubes™ and covered with a 0.5% by volume aqueous solution of (3-aminopropyl)trimethoxysilane, APTMS, (Aldrich). The slides were soaked in this solution for 24 hours, rinsed vigorously with water to remove unreacted and physisorbed APTMS. The slides were then placed individually in 50 mL plastic Digitubes™ and covered with colloid. The slides were allowed to soak in the colloid for 2 hours. The immobilized gold coated slides were then oven driven for 30 minutes and then allowed to cool in a desiccator.

Two substrates were obtained from Prof. Richard van Duyne of Northwestern Univ. with a periodic particle array (PPA) designed for a resonance at 650nm. These samples were prepared using the procedures described previously by Prof. van Duyne.[7] Each substrate was spotted with 5 µL of a saturated naphthlene in ethanol solution and 2 x 10^7 CFU/mL suspension of *B. subtilis* in separate areas on the substrate.

Three separate 15-18 hour overnight cultures of *B. subtilis* (Bacillus subtilis var. niger (aka Bacillus globigii) from Dugway, lot unknown, Bacillus globigii from Dugway, lot 10-37) and *E. coli* were grown in 10 mL of LB media (Sigma Life Science, St. Louis, Mo) in 30 mL glass tubes. A 1 mL aliquot of each separate culture was used to take an optical density. One mL of each separate culture was used to do viable count by spread plate method and 1 mL of each culture was used for Raman spectroscopy.

Bacterial samples were prepared in the following manner to eliminate fluorescence interference from the growth medium. A 1 mL aliquot of an overnight bacterial culture was placed into a sterile 1.5 mL microcentrifuge tube. The sample was pelleted by microcentrifugation at 14,000 rpm for 20 seconds and the supernatant was poured off. The pellet was washed twice by resuspending in 1 mL of sterile phosphate buffered saline (PBS) and repeating centrifugation. A third wash was accomplished using sterile distilled water and the samples were resuspended in 1 mL of sterile distilled water. 0.500 mL of the concentrated washed bacteria was added to 2.500 mL of gold colloid. 0.500 mL of the concentrated washed bacteria was used to do a viable count by spread plate method.

RESULTS AND DISCUSSION

BIOLOGICAL GROWTH

B. subtilis is a gram-positive, nonmotile rod, and creates a spore under unfavorable environmental conditions. *Bacillus subtilis* (*B. subtilis*) is considered non-pathogenic but does cause food spoilage. *B. subtilis* is used to test biological warfare detection systems as a test agent, due to its similarity to biological threat agents. *Escherichia coli*, a gram-negative rod, well established in the literature, was used as a test agent for some of the experiments.

The gram-positive bacteria have a very thick covering on the outside of their membrane made of peptidoglycan. Peptidoglycan is a polymer made up of monomers of N-acetyl muramic acid (NAM) and N-acetyl glutamic acid (NAG) with amino acids attached. These amino acids are then cross-linked with peptide bonds to the chain next to it. This makes it a very strong molecule and provides the cells with structure and protection from osmotic pressure. Up to 80% of a gram-positive cell's mass can be due to the peptidoglycan content. The thickness of the cell wall in gram-positive bacteria results from the crosslinking of five amino acids between the NAM-NAG polymer chains. The outer surface of *B. subtilis* consists of a thick layer of peptidoglycan and ribitol phosphate polymer teichoic acids, as well as, some surface proteins. The outer surface molecules contribute to a net negative charge on the surface of *B. subtilis*.

The outer surface of viable *E. coli* cells consists of an outer bilipid membrane covering a very thin peptidoglycan layer, above an inner bilipid membrane. Peptidoglycan contributes to about 5% of the biomass of a gram-negative cell. The crosslinking in gram-negative bacteria results from one amino acid on one NAM-NAG polymer chain directly crosslinking to an amino acid on an adjacent chain. In addition to the peptidoglycan, the outer membrane of *E. coli* has associated with it: lipopolysaccharides (LPS), peritrichous flagella, and numerous membrane proteins. Like the gram-positive *B. subtilis,* these outer surface molecules, proteins and polymers, contribute to a net negative charge on the outer surface of *E. coli* bacterial cells.

The outer surfaces of both gram positive and gram-negative cells possess a net negative charge. The results support that repelling occurs between the bacteria and the colloidal gold particles, which also possess a net negative charge. Several approaches are now focused on designing a matrix, which will allow the colloidal particles to come into close proximity to the bacterial surface.

COLLOIDAL SERS

The choice of medium appears to be a critical factor in obtaining SERS spectra of bacteria. Visible absorption spectra of the colloids were used to choose a particle size that produced a sufficient plasmon resonance near the laser excitation wavelength of 647.1 nm. SERS spectra of a .01M aqueous solution of pyridine and a 6 mM aqueous solution of rhodamine 6G (R6G) were used to determine which colloid generated the largest enhancement. The SERS enhancement factor, *G*, as defined by Rivas *et al.* can be determined by the following equation:

$$G = \frac{I_{SERS} C_{Aq}}{I_{Aq} C_{SERS}}$$

where I_{SERS} and I_{Aq} are the integrated intensity from 984.6 – 1017.5 cm^{-1} in the SERS and normal Raman spectra of the aqueous solution.[9] These colloids produced a maximum enhancement factor of 24 for pyridine and 50 for R6G. While the 60 nm colloidal solutions displayed a stronger absorbance near the

Krypton laser excitation line, the ~40 nm colloidal solutions produced the greatest enhancement. The 40 nm gold colloids were then used for further SERS studies of bacterial organisms.

Table 1. Gold Colloid Particle Size as a Function of Sodium Citrate Concentration

Concentration of Sodium Citrate	Average Particle Size	Variance
6.25 mM	91 nm	0.037
12.5 mM	39 nm	0.046
25 mM	26 nm	0.024
50 mM	25 nm	0.040
100 mM	25 nm	0.047

IMMOBILZED GOLD COLLOID

Gold colloids were prepared and immobilized on quartz slides to generate a SERS active surface. Gold colloidal suspensions were made using a solution of hydrogen tetrachloroaurate (III) hydrate and distilled water with the addition of a sodium citrate solution. To activate the substrate surface for immobilzation, quartz slides were fully hydrolized using a piranha solution (H_2SO_4:H_2O_2, 4:1) and subsequently derivatized in a 4:1 solution of methanol or amino propyl tri-methoxysilane (APTMS) or mercapto-propyl tri-methoxysilane (MPTMS). The quartz slides were then soaked in a colloidal gold suspension for varying lengths of time.

The slides were initially soaked resting crosswise in a Digatube™. However, particle settling was occurring which resulted in uneven distribution of particles between on the slides. This problem was resolved by placing one slide in each Digatube™ and continually shaking (130 rpm) the tubes during the duration of the soaking. After the slides were removed from soaking in the suspension, they were dried in a 75 °C oven for 15 minutes.[10] The weight percent of gold on each slide was measured using an X-Ray [illegible]escence Spectrometer (XFS) and spectra of the slides from the UV-visible spectrometer were used to determine the plasmon resonance. The particle size of the gold was determined using dynamic light scattering at 488 nm (Brookhaven, BI-HV). The formation of roughened metal islands on the surface is critical to generating a strong SERS effect. Atomic Force Microscopy (AFM) was utilized to characterize the slide surfaces (Figure 1).

Representative gram-positive and gram-negative bacteria were selected for this SERS study. Initial efforts were aimed at obtaining reproducible spectra representative of each sample. These efforts were hampered by variability and stability of the commercial colloidal solutions themselves. While this may preclude the use of colloidal solutions for a field-deployable detection scheme, it did not prevent their use in these preliminary studies. Figure 2 shows spectra of *B. Subtillis* and *E. Coli* from 40 nm colloidal gold. Figure 3 shows spectra of several additional gram positive and gram negative bacteria on immobilized colloidal slides. The most dominant feature in the spectrum is the band at 1210 cm^{-1} most likely attributed to the peptidoglycan layer. What is strikingly absent in these spectra are the dominant amide I and II bands observed in UV Resonance Raman Spectra.[11]

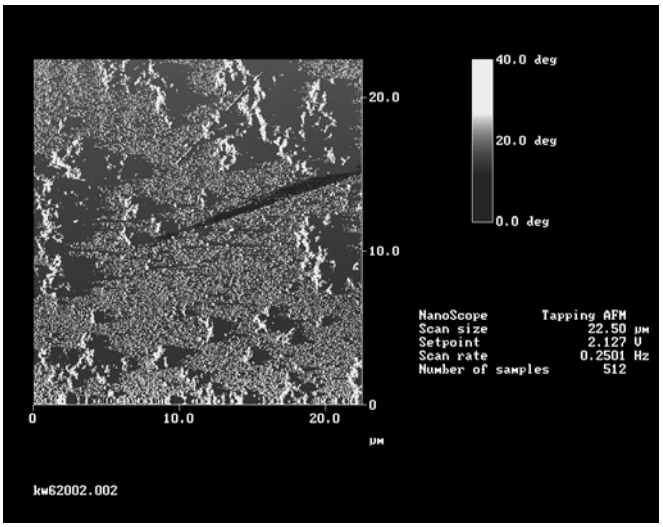

Figure 1. AFM Image of Gold Aggregation on Slide Surface

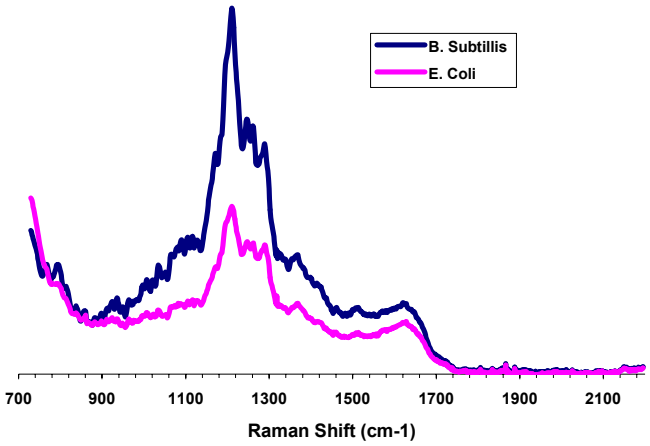

Figure 2. SERS Spectra of Gram Positive and Gram Negative Bacteria

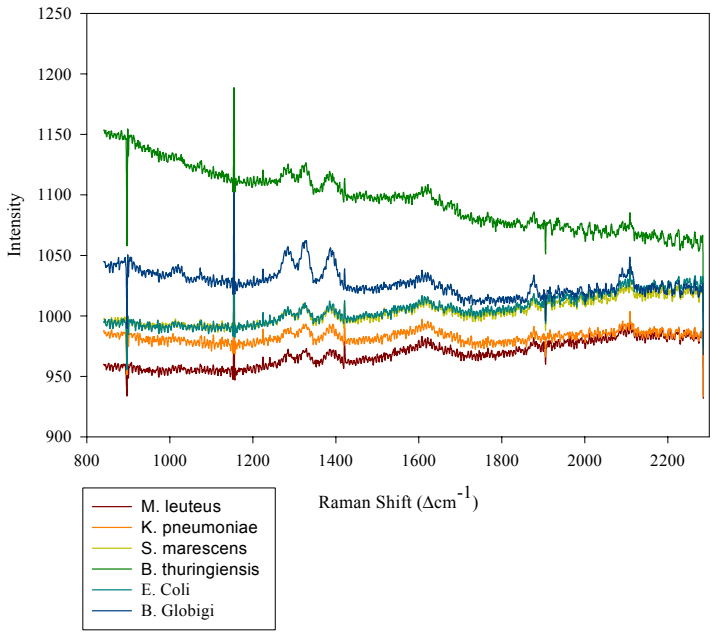

Figure 3. SERS Spectra of Bacteria on Immobilized Gold Colloid

SERS SUBSTRATES

The two PPA substrates have been evaluated for their SERS performance OTHER. Absorption spectra were collected from 400 nm to 850 nm. The resonance maximum of these substrates was observed at ~675 nm while the SERS excitation wavelength was 647.1 nm. The SERS spectra collected from these substrates did not show a detectable SERS signal from the bacteria or naphthalene, although a strong glass signal was observed. This could be due to the mismatch between the excitation wavelength and the resonance wavelength. Another possible explanation could be the need for a surface coating to negate the surface charge of the metal particles that make up the PPA and may act to repel the bacterial particles from the surface, keeping them outside the optimum range for SERS enhancement.

CONCLUSIONS

While the detection of bacteria using SERS possible, our preliminary results indicate spectral differences in gram-positive and gram-negative bacteria are based on the thickness of the peptidoglycan layer. The amide bands weak or absent in SERS spectra of vegetative cells making speciation of the bacteria difficult.

Electrostatic effects between the negatively charged bacteria and negatively charged metal particles difficult to overcome, making substrate preparation the key to obtaining reproducible SERS spectra. While our preliminary examination of SERS substrates is still underway, SERS spectra have been collected using colloidal Au particles and immobilized colloid on quartz. An enhancement factor of 24 has been observed and a limit of detection of 1 x 10^4 CFU/mL has been projected. The use of APTMS or MPTMS to immobilize the colloid had negligible differences in gold aggregation and SERS activation. Based on data of the time the slides spent soaking in the colloid, it was determined that longer soaking times increased aggregation on slides and did not augment the mass percent of gold per slide or the SERS activation. As a result, slides were later left in suspension for no more than two hours. In preparing the

gold colloids, varying the sodium citrate volumes did not alter particle size. However, differing concentrations of sodium citrate did produce various sizes of colloidal particles. From the SERS spectra of the Rhodamine 6-G, it was found that a 12.5 mM solution of sodium citrate produced 40 nm particles, which produced the greatest amount of SERS activity.

PPA substrates have been examined and no SERS signal has yet been observed. Since these results are still quite preliminary, the PPA substrates may prove advantageous with further development. Studies are also underway to determine the need for surface coatings to negate the repulsive charge effects between the negatively charged bacteria and the negatively charged metal particles or surfaces.

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